



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(54) Title: PROCESS FOR THE PREPARATION OF ACANTHOIC ACID AND PHARMACEUTICAL COMPOSITION COMPRISING SAME</p>												
<p>(57) Abstract</p> <p>Process for the preparation of (-)-pimara-9(11), 15-diene-19-oic acid(acanthoic acid) and pharmaceutical compositions comprising acanthoic acid useful for the treatment of diseases caused by an excessive production of interleukin-1 or tumor necrosis factor-<math>\alpha</math>.</p> <div data-bbox="695 1121 1435 1843"> <table border="1"> <caption>IL-1 production (% of control) vs concentrations (μg/ml)</caption> <thead> <tr> <th>concentrations (μg/ml)</th> <th>IL-1 production (% of control)</th> </tr> </thead> <tbody> <tr> <td>0.1</td> <td>100</td> </tr> <tr> <td>1</td> <td>68</td> </tr> <tr> <td>10</td> <td>38</td> </tr> <tr> <td>100</td> <td>35</td> </tr> </tbody> </table> </div>			concentrations (μg/ml)	IL-1 production (% of control)	0.1	100	1	68	10	38	100	35
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PROCESS FOR THE PREPARATION OF ACANTHOIC ACID  
AND PHARMACEUTICAL COMPOSITION COMPRISING SAME

FIELD OF THE INVENTION

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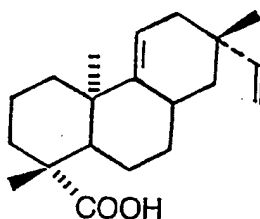
The present invention relates to a process for the preparation of acanthoic acid((-)-pimara-9(11),15-diene-19-oic acid) and pharmaceutical compositions comprising same useful for the treatment of diseases caused by an excessive  
10 production of interleukin-1(hereinafter, referred to as "IL-1") or tumor necrosis factor- $\alpha$ (hereinafter, referred to as "TNF- $\alpha$ ").

BACKGROUND OF THE INVENTION

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Acanthopanax koreanum Nakai(Araliaceae), which is found indigenously in Cheju Island, the Republic of Korea, has been used traditionally as remedies for neuralgia, paralysis, lumbago, etc. Various useful components,  
20 including acanthoic acid of the following formula (A), have been isolated from its root bark(Kim, Y. H. and Chung, B. S., J. Nat. Pro., 51, 1080(1988)).

25



(A)

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Acanthoic acid has been reported to have various pharmacological effects, e.g., analgesic and anti-inflammatory activity which is due to its ability to inhibit the leukocyte migration and prostaglandin  $E_2$ (PGE<sub>2</sub>) synthesis,  
35 and to exhibit very low toxicity, e.g., 1000 mg/kg of minimum lethal dose(MLD) when administered to a rat(Lee, Y. S., "Pharmacological Study for (-)-Pimara-9(11),15-Diene-19-

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oic Acid, A Component of Acanthopanax koreanum Nakai", doctorate thesis, Dept. of Pharmacy, Seoul National University, Korea, 1990).

As is well known, IL-1 is a regulatory factor which participates in a wide range of human defensive and immune mechanisms(see, e.g., Dinarello, D. A., FASEB J., 2, 108 (1988)). IL-1, first discovered to be produced by activated macrophages, is produced and secreted by various cells, e.g., fibroblasts, keratinocytes, T cells, B cells, and astrocytes of brain; and has been reported to have various functions including: stimulating the proliferation of CD4+T cells(Mizel, S. B., Immunol. Rev., 63, 51(1982)); stimulating the cell-killing effect of thymic T<sub>c</sub> cells through its binding to a T cell receptor(TCR)(McConkey, D. J., et al., J. Biol. Chem., 265, 3009(1990)); inducing the production of various materials participating in the inflammatory mechanisms, e.g., PGE<sub>2</sub>, phospholipase A<sub>2</sub>(PLA<sub>2</sub>), and collagenase(Dejana, E., et al., Bolid, 69, 695-699 (1987)); inducing the production of acute-phase proteins in liver(Andus, T., et al., Eur. J. Immunol., 123, 2928(1988)); raising blood pressure in the vascular system(Okusawa, S., et al., J. Clin. Invest., 81, 1162(1988)); inducing the production of other cytokines, e.g., IL-6 and TNF(Dinarello, C. A., et al., J. Immunol., 139, 1902(1987)), etc.

As has been reported, IL-1 relates to various immune diseases, e.g., rheumatoid arthritis(Nouri, A. M., et al., Clin. Exp. Immunol., 58, 402(1984)), rejection mechanisms after the kidney transplantation(Mauri and Teppo, Transplantation, 45, 143(1988)), and septicemia(Cannon, J. G., et al., Lymphokine Res., 7, 457(1988)). IL-1 has been also reported to induce a fever and pain when administered in a large amount to a human body(Smith, J., et al., Am. Soc. Clin. Oncol., 9, 710(1990)).

TNF- $\alpha$ , first discovered in a serum of an animal treated with BCG(Bacille Calmette-Guérin) or LPS(lipopolysaccharide) (Carswell, E. A., et al., Proc. Natl. Acad. Sci. U.S.A., 72, 3666(1975)), has been reported to be produced by various

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cells, e.g., activated macrophages and fibroblasts. Further, TNF- $\alpha$  has been reported to have the functions of: killing the fibrosarcoma L929 cells(Espevik and Nissen-Meyer, J. Immunol. Methods, 95, 99(1986)); stimulating the  
5 proliferation of fibroblasts(Sugarman, B. J., et al., Science, 230, 943(1985)); inducing the production of PGE<sub>2</sub>, arachidonic acid, etc. which may be involved in inflammatory responses(Suttys, et al., Eur. J. Biochem., 195, 465(1991)); inducing the production of IL-6 or other growth factors(Van  
10 Hinsbergh, et al., Blood, 72, 1467(1988)), etc.

TNF- $\alpha$  has been also reported to participate, directly or indirectly, in various diseases; and examples of said diseases are: infectious diseases carried by trypanosoma, strains of the genus Plasmodium, etc.(Cerami, A., et al.,  
15 Immunol. Today, 9, 28(1988)); autoimmune diseases such as systemic lupus erythematosus(SLE) and arthritis(Fiers, W., FEBS, 285, 199(1991)); AIDS(Mintz, M., et al., Am. J. Dis. Child., 143, 771(1989)); septicemia(Tracey, K. J., et al., Curr. Opin. Immunol., 1, 454(1989)); and infections  
20 (Balkwill, F. R. 1989, Cytokines in Cancer Therapy, Oxford University Press).

These observations have buttressed the importance of regulating the production of IL-1 and TNF- $\alpha$  for the maintenance of the homeostasis of immune system in a human  
25 body and for the treatment and prophylaxis of related diseases.

Accordingly, there have been proposed numerous approaches to regulate the production of interleukins. For instance, it has been reported that the occurrence of  
30 septicemia, arthritis, inflammations, etc. in animal models can be decreased by the inhibition of IL-1 binding to its receptors by employing naturally occurring IL-1 receptor inhibitors(IL-1 Ra)(Dinarello, C. A. and Thompson, R. C., Immunol. Today, 12, 404(1991)), and there have been proposed  
35 certain methods for inhibiting the activity of IL-1 by employing particular antibodies(Giovine, D. F. S. and Duff, G. W., Immunol. Today, 11, 13(1990)). In case of IL-6,

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proliferation of myelocytes in a patient suffering from myeloma which is caused by an excessive secretion of IL-6 has been suppressed by employing antibodies against IL-6 or IL-6 receptor (Suzuki, H., Eur. J. Immuno., 22, 1989(1992)).

5        However, no substance or method has been reported to inhibit specifically the productions of IL-1 and TNF- $\alpha$  and, therefore, efforts have continued for the discovery of specific inhibitors against the production of IL-1 and TNF- $\alpha$ .

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#### SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a process for the preparation of acanthoic acid from root bark of Acanthopanax koreanum Nakai.

15        Another object of the present invention is to provide a pharmaceutical composition comprising a therapeutically effective amount of acanthoic acid and a pharmaceutically acceptable carrier, which is useful for the treatment of  
20        immune diseases caused by an excessive production of IL-1 or TNF- $\alpha$ .

#### BRIEF DESCRIPTION OF THE DRAWINGS

25        The above and other objects and features of the present invention will become apparent from the following description of the invention taken in conjunction with the accompanying drawings, in which:

30        Fig. 1 shows the cytotoxicity of acanthoic acid on human monocytes and macrophages;

Fig. 2 depicts the inhibitory effect of acanthoic acid on the production of IL-1 in human monocytes and macrophages;

35        Fig. 3 provides the inhibitory effect of acanthoic acid on the production of TNF- $\alpha$  in human monocytes and macrophages;

Fig. 4 offers the inhibitory effect of acanthoic acid

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on the production of TNF- $\alpha$  in rat alveolar macrophages and lymphocytes;

Fig. 5 presents the inhibitory effect of acanthoic acid on the production of the reactive oxygen species in human monocytes and macrophages;

Fig. 6 illustrates the inhibitory effect of acanthoic acid on the proliferation of NIH3T3 fibroblasts;

Fig. 7 displays the inhibitory effect of acanthoic acid on the production of collagen in rat pulmonary fibroblasts;

Fig. 8 exemplifies the inhibitory effect of acanthoic acid on the production of collagen in rat pulmonary tissues;

Fig. 9 exhibits the inhibitory effect of acanthoic acid on the silicosis in rat;

Fig. 10 demonstrates the effect of acanthoic acid on GOT and GPT level in serum of a rat suffered from induced hepatocirrhosis; and

Fig. 11 highlights the effect of acanthoic acid on rat hepatocirrhosis.

## 20 DETAILED DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

In accordance with the present invention, it has been found that acanthoic acid extracted from Acanthopanax koreanum Nakai possesses the ability to specifically inhibit the production of IL-1 or TNF- $\alpha$ ; and, therefore, a pharmaceutical composition comprising an effective amount of acanthoic acid is useful for the treatment of various immune diseases caused by an excessive production of IL-1 or TNF- $\alpha$ .

Said acanthoic acid may be extracted from the root bark of A. koreanum Nakai by employing various organic solvents, e.g., methanol, diethyl ether, or a mixture thereof, etc. Especially, acanthoic acid may be prepared in accordance with the following preferred embodiment of the present invention.

To 1kg of dried root bark of A. koreanum Nakai is added

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1 to 3l, preferably 2l of methanol; and the mixture is heated at a temperature ranging from 20 to 60°C, preferably, at a room temperature, for at least 10 hours, preferably, for 12 hours, and filtered. Said procedure is repeated,  
5 preferably three times, and the combined filtrates are concentrated under a reduced pressure to obtain a methanol extract.

100g of said methanol extract is partitioned with 200 to 400ml, preferably 300ml, of water and 200 to 400ml, preferably 300ml, of diethyl ether. The diethyl ether fraction is separated therefrom and then concentrated under a reduced pressure to obtain a diethyl ether extract. Said extract is purified by a silica gel column chromatography using a mixture of hexane and ethyl acetate as an eluent to  
15 obtain acanthoic acid.

Said acanthoic acid exhibits anti-inflammatory and anti-fibrogenic effects, inhibits the synthesis of collagen and the production of the reactive oxygen species, and reduces GOT and GPT levels in serum. That is, it can be employed in a pharmaceutical composition for the treatment of immune diseases caused by an excessive production of IL-1 or TNF- $\alpha$ , e.g., septicemia, rheumatoid arthritis, inflammation, hepatocirrhosis and silicosis.

The pharmaceutical compositions of the present invention may comprise pharmaceutically acceptable excipients, carriers or diluents in addition to acanthoic acid as an active ingredient. The pharmaceutical formulations may be prepared in accordance with any of the conventional procedures.

30 In preparing the compositions, the active ingredient is preferably admixed or diluted with a carrier, or enclosed within a carrier, which may be in the form of a capsule, sachet or other container. When the carrier serves as a diluent, it may be a solid, semi-solid or liquid material acting as a vehicle, excipient or medium for the active  
35 ingredient. Thus, the compositions may be in the form of a tablet, pill, powder, sachet, elixir, suspension, emulsion,



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solution, syrup, aerosol, soft and hard gelatin capsule, sterile injectable solution, sterile packaged powder and the like.

Examples of suitable carriers, excipients, and diluents are lactose, dextrose, sucrose, sorbitol, mannitol, starch, gum acacia, alginate, calcium phosphate, calcium silicate, gelatin, cellulose, methyl cellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, talc, magnesium stearate and mineral oil.

The formulations may additionally include lubricating agents, wetting agents, flavoring agents, emulsifiers, preservatives and the like. The compositions of the invention may be formulated so as to provide quick, sustained or delayed release of the active ingredient after their administration to a patient by employing any of the procedures well known in the art.

The pharmaceutical compositions can be administered by a variety of routes including oral, transdermal, subcutaneous, intravenous and intramuscular introduction. A typical daily dose of the active ingredient may range from about 1 to 500  $\mu\text{g/kg}$  body weight, preferably 30 to 300  $\mu\text{g/kg}$  body weight, and can be administered in a single dose or in divided doses. However, it should be understood that the amount of the active ingredient actually administered ought to be determined in light of various relevant factors including the condition to be treated, the chosen route of administration, the age and weight of the individual patient, and the severity of the patient's symptom; and, therefore, the above dose should not be construed to limit the scope of the invention in any way.

The following Preparation Example and Examples are intended to further illustrate the present invention without limiting its scope; and the experimental methods used in the Examples can be practiced in accordance with the Reference Examples given herein below, unless otherwise stated.

Further, percentages given below for solid in solid

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mixture, liquid in liquid, and solid in liquid are on a wt/wt, vol/vol and wt/vol basis, respectively, unless specifically indicated otherwise.

5 Preparation Example: Preparation of Acanthoic Acid

About 1.7kg of well-dried root bark of A. koreanum Nakai was chopped and extracted with about 4ℓ of methanol at a room temperature for 24 hours, and then filtered. The  
10 extraction procedure was repeated three times and the combined filtrates were concentrated under a reduced pressure to obtain about 200g of the methanol extract.

All of said methanol extract was partitioned with 600ml of distilled water and 600ml of diethyl ether. Diethyl  
15 ether layer was separated and concentrated under a reduced pressure to obtain 110g of diethyl ether extract.

All of said diethyl ether extract was purified by a silica gel column chromatography using hexane:ethyl acetate (20:1(v/v) → 5:1(v/v)) to obtain about 30g of active  
20 material in a yield of 1.76%.

The active material was identified as acanthoic acid by using thin layer chromatography(TLC) compared with the standard(Kim, Y. H. and Chung, B. S., J. Nat. Pro., 51, 1080(1988)), and this was also confirmed by <sup>1</sup>H-NMR, <sup>13</sup>C-NMR,  
25 MS and IR.

Reference Example 1: Separation of Cells for Assay

(1) Separation of human monocytes, macrophages and  
30 neutrophils

Normal human peripheral blood was heparin-treated and diluted with equal amount of Hank's balanced salt solution (HBSS: Ca<sup>2+</sup> and Mg<sup>2+</sup> free). The diluted blood was put into  
35 a centrifuge tube containing therein Ficoll-Hypaque(Sigma, St. Louis, MO, U.S.A.) layer having a density of 1.077 piled up on Ficoll-Hypaque layer having a density of 1.119, and

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then centrifuged at 700xg for 30 minutes to obtain monocytes from the layer between Ficoll-Hypaque layer having a density of 1.077 and serum layer, and neutrophils from the layer between Ficoll-Hypaque layer having a density of 1.077 and that having a density of 1.119. The separated cells were washed twice with 4°C HBSS(Ca<sup>2+</sup> and Mg<sup>2+</sup> free) and suspended in RPMI 1640 medium(Gibco, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum(FBS, Hyclone, Logan, Utah, U.S.A.). The suspensions were added to the wells of 24-well incubation plate(Costar, Cambridge, MA, U.S.A.) and incubated at 37°C for 2 hours to obtain monocytes, macrophages and neutrophils.

## (2) Separation of fibroblasts

15

Fibroblasts were separated from rats by using a modification of the method of Phan, S. H., et al. described in J. Clin. Invest., 76, 241(1985), as follows.

A rat was anesthetized with ether and its lungs were isolated on the aseptic worktable. The lungs were cut into small pieces in the size ranging from 2 to 4mm and suspended in phosphate buffered saline(PBS) containing collagenase and 0.5% trypsin to digest the tissues at 37°C for 2 hours. The suspension was filtered through sterilized gauze to remove undigested tissues, etc. The separated cells were washed with PBS twice or three times and suspended into RPMI 1640 medium(Gibco, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum(FBS, Hyclone, Logan, UT, U.S.A.). The suspension was added to the wells of incubation plate and incubated at 37°C for 1 to 2 days in 5% CO<sub>2</sub> incubator (Lunaire Environ, Inc., Pennsylvania, U.S.A.). The plate was washed with RPMI 1640 medium to remove the cells which did not adhere to the plate. Fresh medium was added to the plate and the incubation was continued until the confluent layer was formed. The cells undergone subcultures less than 5 times were used in the following tests.

NIH3T3 fibroblast(ATCC CRL 1658) was cultured in RPMI

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1640 medium containing 10% FBS under the same conditions as described above.

(3) Treatment of cells with acanthoic acid

5

Acanthoic acid was added in various concentrations to  $5 \times 10^5$ /ml of cells obtained in the above procedures, and the cells were precultured at 37°C for 1 hour in 5% CO<sub>2</sub> incubator. Then, 1ml each of silica (100 µg/ml) and RPMI 1640  
10 medium containing 2% FBS were added thereto and the cells were cultured under the same conditions as above for 48 hours. The culture supernatant was collected and centrifuged at 1,500 rpm for 10 minutes to remove the cells and silica. The obtained supernatant was dialyzed against  
15 PBS and filtered by 0.2 µm filtration syringe, and the filtrate was stored at -20°C.

Reference Example 2: Assay for Cytotoxicity of Acanthoic Acid

20

The cytotoxicity of acanthoic acid was determined by the following procedures.

According to the procedures of Reference Example 1 (3),  $5 \times 10^5$  cells/ml each of monocytes and macrophages obtained in  
25 Reference Example 1 (1) were treated with 0.1, 1, 10 or 100 µg/ml of acanthoic acid obtained in the Preparation Example and incubated under the same conditions. In accordance with the method of Alley, M.C., et al. described in Cancer Res.,  
30 48, 589(1988), each culture was added to the wells of the incubation plate in an amount of 1ml/well, and 0.5mg of 3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each of the wells. After incubating at  
37°C for 4 hours, the culture was centrifuged to remove supernatant. 100 µl each of acidified isopropanol (0.04N HCl  
35 in isopropanol) was added to the cells in each well to elute formazan produced by the living cells, and optical density (O.D.) was determined at 540nm by using an ELISA reader

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(Titertek multiskan Mcc/340)(Fig. 1).

Fig. 1 shows the relative values of optical density of the sample with respect to the concentration of acanthoic acid when the optical density of the control group which was not treated with acanthoic acid is regarded as 100%. When the survival rate of monocyte and macrophage decreases due to the toxicity of acanthoic acid, the production of formazan also decreases, which causes the optical density to decrease. The samples treated with acanthoic acid show no significant difference from the control group until the concentration of acanthoic acid reaches 10 $\mu$ g/ml. Therefore, it is confirmed that acanthoic acid has no cytotoxicity at the concentration lower than 10 $\mu$ g/ml and, hereinafter, all the tests were carried out in this concentration range.

Example 1: Inhibition of IL-1 Production in Human Monocytes and Macrophages by Acanthoic Acid

The monocytes and macrophages obtained in Reference Example 1 (1) were incubated with 0.1 to 100 $\mu$ g/ml of acanthoic acid for an hour and treated with 100 $\mu$ g/ml of silica for 48 hours. The culture was centrifuged to obtain supernatant, which was then dialyzed against PBS. The activity of IL-1 in the dialyzate was determined in accordance with the method of Gery described in Cellular Immunology, 64, 293-303(1981) as follows.

1x10<sup>7</sup> cells/ml of C3H/HeJ mouse thymocytes suspended in RPMI 1640 medium containing 10% FBS were treated with 1 $\mu$ g/ml of phytohemagglutinin(PHA, Burroughs Wellcome, Research Triangle Park, NC, U.S.A.) and 100 $\mu$ l each of the suspensions was added to the wells of 96-well incubation plate(Costar, plat-bottomed). 50 $\mu$ l of said dialyzate and RPMI 1640 medium containing 10% FBS were added to each of the wells. Then, the plate was incubated at 37°C under 5% CO<sub>2</sub> for 72 hours. At 16 hours before the completion of the incubation, 0.5  $\mu$ Ci/well of <sup>3</sup>H-thymidine was added to the wells. When the incubation was completed, the cells were collected on the

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glass fiber filter and the amount of incorporated  $^3\text{H}$ -thymidine was determined by liquid scintillation counter(Beckman).

Fig. 2 shows relative values of the amount of incorporated  $^3\text{H}$ -thymidine with respect to the concentration of acanthoic acid when the amount of incorporated  $^3\text{H}$ -thymidine of the control group which was not treated with acanthoic acid is regarded as 100%. As can be seen from Fig. 2, the productions of IL-1 in human monocytes and macrophages were inhibited by acanthoic acid in a concentration-dependent mode.

Example 2: Inhibition of TNF- $\alpha$  Production in Human Monocytes and Macrophages by Acanthoic Acid

15

Human monocytes and macrophages were treated with acanthoic acid in accordance with the same procedures as in Example 1, and the activity of TNF- $\alpha$  was measured by cell lytic assay in accordance with the method of Aggarwal as described in J. Biol. Chem., 260, 2345(1985)).

TNF- $\alpha$  dependent L929 fibroblasts(ATCC CCL1) were suspended in RPMI 1640 medium containing 5% FBS and added to the wells of 96-well incubation plate in an amount of  $3 \times 10^4$  cells/well. The plate was incubated at 37°C for 2 hours to make the cells adhere to the incubation plate. Then, the medium was removed and 1 $\mu\text{g}/\text{ml}$  of actinomycin D(Sigma) and 50 $\mu\text{l}$  of culture dialyzate of monocytes and macrophages obtained in Example 1 were added to each of the wells. The final concentration of FBS in each well was adjusted to 5% and the cells were incubated at 37°C under 5%  $\text{CO}_2$  for 24 hours.

When the incubation was completed, the medium was removed and the cells were washed twice with PBS and stained for 5 minutes with 0.5% crystal violet solution in 20% methanol. The cells were washed three times with PBS and dried. Then, 100 $\mu\text{l}$  of 33% acetic acid was added to each of the wells to release the dye, and then the O.D. of sample

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obtained from each of the wells was determined by using ELISA reader with 570nm reading filter and 405nm reference filter. The amount of released dye was calculated from the O.D. value with reference to that of the internal control group, i.e., rHu TNF- $\alpha$ (Genzyme).

As can be seen from Fig. 3, 5 $\mu$ g/ml or more of acanthoic acid inhibits the production of TNF- $\alpha$  by at least 90%.

Example 3: Inhibition of TNF- $\alpha$  Production in Rat Alveolar Macrophages and Lymphocytes by Acanthoic Acid

A rat was anesthetized with ketamine and its alveolar macrophages and lymphocytes were obtained therefrom by inserting a sterilized thin tube into the branchia and repeating three times the injection and sucking out of 10ml of RPMI 1640 medium with a 30ml syringe. The obtained cells were centrifuged at 400xg for 5 minutes, suspended in 50ml of RPMI 1640 medium containing 10% FBS and then incubated at 37°C for 2 hours to adhere to the incubation plate. The plate was washed twice with PBS to remove floating cells and obtain alveolar macrophages and lymphocytes.

Each 2 x 10<sup>5</sup> cells/well of the alveolar macrophages and lymphocytes were added to the wells of 24-well incubation plate and 10 $\mu$ g/ml of acanthoic acid was added to each of the wells. The cells were precultured at 37°C for 1 hour and treated with 100 $\mu$ g/ml of silica for 3 days. The culture was dialyzed against PBS and the activity of TNF- $\alpha$  in the dialyzate was determined by using TNF- $\alpha$  dependent L929 cell line in accordance with the procedure as described in Example 2.

As can be seen from Fig. 4, it was observed that the production of TNF- $\alpha$  in rat alveolar macrophages and lymphocytes was also inhibited by acanthoic acid. In Fig. 4, med, Si and Si+acan. represent non-treated control group, silica-stimulated sample and acanthoic acid-treated and silica-stimulated sample, respectively.

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Example 4: Inhibition of Production of Reactive Oxygen  
Species by Acanthoic Acid

Inflammatory responses are known as a cascade reaction comprising the secretion of various cytokines, e.g., IL-1, from immune cells stimulated by various stimulants; production of phospholipase A<sub>2</sub>, lysosomal enzyme, reactive oxygen species, etc. by other immune cells stimulated by said cytokines; and damage of tissues induced by the above products (Pruzanski, W. and Vadas, P., Immunol. Today, 12, 143(1991)). The ability of acanthoic acid to block the inflammatory reactions was tested by measuring their inhibitory activity to the production of reactive oxygen species, i.e., H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>.

The amount of H<sub>2</sub>O<sub>2</sub> was determined by a microassay employing 96-well microplate as follows. 5 x 10<sup>5</sup> cells of neutrophils were added to each of the wells containing RPMI 1640 medium, and then 25μl of horseradish peroxidase (500μg/ml; type II, Sigma) and 75μl of phenol red (1mg/ml) were added to each of the wells. Thereafter, the cells were treated with 10, 20 and 50μg/ml of acanthoic acid for an hour, stimulated with 10<sup>-7</sup> M phorbol myristate acetate (PMA) and then incubated at 37°C for 60 minutes. When the incubation was completed, 3M NaOH was added to the wells in an amount of 25μl/well to stop the reaction and O.D. was measured at 620nm by using ELISA reader (Dynatech Lab. Inc.) to determine the change of colors with respect to the oxidation of phenol. The amount of H<sub>2</sub>O<sub>2</sub> was determined by employing a standard curve prepared by diluted H<sub>2</sub>O<sub>2</sub> (Sigma).

For the purpose of measuring the amount of produced O<sub>2</sub><sup>-</sup>, neutrophils suspended in RPMI 1640 medium in a concentration of 1 x 10<sup>6</sup> cells/800μl was added to a part of the wells of 24-well plate and 10μg/ml of superoxide dismutase (SOD, Sigma) was added to the empty wells. The plate was stored at 37°C for 2 minutes, and cytochrome C (3mg/ml, Sigma) was added to the wells in a concentration of 100μl/well. The cells were treated with 10, 20 and 50μg/ml of acanthoic acid



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for an hour and reacted at 37°C for 20 minutes by introducing  $10^{-7}$  PMA as a stimulant. The reaction was terminated by adding 1mM N-ethylmaleimide(Sigma) to the wells and the culture was centrifuged at 1,600xg for 10 minutes to obtain a supernatant. The change of color of the supernatant caused by the reduction of cytochrome C was measured at 550nm by using a UV-Visible spectrophotometer (Kontron Instrument, Milano, Italy). The amount of produced  $O_2^-$  was represented by the concentration of SOD which can suppress the reduction of cytochrome C in  $1 \times 10^6$  cells for 20 minutes, by employing the extinction coefficient of cytochrome C ( $E_{550nm} = 1.83 \times 10^4 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

As can be seen from Table I, 50  $\mu\text{g/ml}$  of acanthoic acid inhibits the production of  $H_2O_2$  by 85%, and the production of  $O_2^-$  by 72%. The result shows that acanthoic acid has a strong inhibitory activity to the inflammatory response.

Table I: Inhibitory effect of acanthoic acid on the production of reactive oxygen species in human neutrophils

Sample	Amount of produced reactive oxygen species (% to the control group)	
	$H_2O_2$ (nM/60 min.)	$O_2^-$ (nM/20 min.)
Medium only	11.5(11.1)	2.0(12.9)
PMA	103.6(100)	15.5(100)
PMA + Acanthoic acid		
10 $\mu\text{g/ml}$	72.6(70)	11.9(76.8)
20 $\mu\text{g/ml}$	38.7(37.4)	4.9(31.6)
50 $\mu\text{g/ml}$	16.4(15.8)	4.4(28.4)

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On the other hand, the same procedures as above were repeated to determine the amount of  $H_2O_2$  and  $O_2^-$  produced by  $5 \times 10^5$  cells of human monocytes and macrophages which was treated with  $10\mu g/ml$  of acanthoic acid at  $37^\circ C$  for an hour and then stimulated by  $100\mu g/ml$  of silica. As can be seen from Fig. 5, the amounts of  $H_2O_2$  and  $O_2^-$  decrease significantly in the acanthoic acid-treated and silica-stimulated monocytes and macrophages(si+acan.) in contrast with the control group treated with only silica(si+med).

Example 5: Inhibition of Proliferation of Fibroblasts by Acanthoic Acid

Fibrosis is caused largely by the proliferation of fibroblasts or by the synthesis of collagen. To confirm the effect of acanthoic acid on the proliferation of fibroblasts, human monocytes and macrophages were treated with  $10\mu g/ml$  of acanthoic acid and cultured according to the procedure as described in Reference Example 1 (3). The culture was dialyzed against PBS, and  $50\mu l$  of the dialyzate was added to the wells of an incubation plate.  $5 \times 10^3$  Cells of NIH3T3 fibroblasts(ATCC CRL 1658) were added to each of the wells and then cultured at  $37^\circ C$  under  $5\%$   $CO_2$  for 5 days. After the addition of  $^3H$ -thymidine to the culture, the cells were cultured for additional 16 hours. The amount of  $^3H$ -thymidine incorporated in the cell was determined by employing liquid scintillation counter(Fig. 6). As can be seen from Fig. 6, the proliferation of fibroblasts is inhibited significantly in the acanthoic acid-treated and silica-stimulated fibroblasts (si+acan.) in contrast with the control group treated with only silica(si+med). It is supposed that such inhibition is caused by the action of acanthoic acid reducing the production of interleukins or other fibrogenic growth factors which cause fibrosis, or inducing anti-fibrogenic factors.

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Example 6: Inhibition of Collagen Synthesis by Acanthoic Acid

IL-1 and TNF- $\alpha$  are known as cytokines which cause fibrosis and induce collagen synthesis in rat fibroblast(Kang, H. S., et al., Korean. J. Immunol., 14, 193(1992)). For the purpose of confirming the ability of acanthoic acid to suppress the action of IL-1 and TNF- $\alpha$ , its inhibitory effect on the collagen synthesis in rat pulmonary fibroblast and pulmonary tissues was determined. The amount of produced collagen in the culture of rat pulmonary fibroblast was measured by an indirect ELISA method, and that of pulmonary tissue was determined by measuring the concentration of hydroxyproline and calculating the amount of collagen therefrom by using the standard curve of internal control group.

To measure the amount of synthesized collagen in the culture of rat pulmonary fibroblast, collagen(Sigma, type I) as an internal control group was dissolved thoroughly in 1M acetic acid containing 1mg/ml of pepsin, and the solution was serially diluted by 5-fold with coating buffer(0.05M carbonate, pH 9.6) in a concentration ranging from 1 $\mu$ g to 16pg. The diluted solutions were added to the wells of flat-bottomed microtiter plate(Dynatech, Immulon 2) in an amount of 100 $\mu$ l/well.

On the other hand, 1 ml of the culture supernatant of rat pulmonary fibroblasts obtained in Reference Example 1 (2) was 10- to 20-fold concentrated by using speed vac dryer(Savant, Hicksville, NY, U.S.A.) and dissolved in 100 $\mu$ l of coating buffer(0.1M NaHCO<sub>3</sub>, 0.02% NaN<sub>3</sub>; pH was adjusted to 9.6 with Na<sub>2</sub>CO<sub>3</sub>) and the solution was added to the wells in an amount of 100 $\mu$ l/well and then coated at 4°C overnight.

The plate was washed three times with washing buffer(PBS, 0.05% Tween 20, pH 7.4), and 1% bovine serum albumin(BSA, Sigma) was added to the wells in an amount of 100 $\mu$ l/well. The plate was incubated at a room temperature for 2 hours to block the uncoated parts. The plate was washed four times with the same buffer as above, and alkaline phosphatase-conjugated

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rabbit anti-goat IgG(Cappel, Durham, NC, U.S.A.), which was 1,000-fold diluted with a dilution buffer(0.05M Tris-HCl, 1mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.15M NaCl, 0.02%  $\text{NaN}_3$ , 1% BSA, 0.05% Tween 20, pH 8.1), was added to the wells in an amount of 100 $\mu\text{l}$ /well.

5        The plate was incubated at 37°C for 2 hours and then washed three times with the same buffer as above. To the wells was added 100 $\mu\text{l}$ /well of p-nitrophenyl phosphate which was diluted with substrate buffer(0.05M  $\text{NaHCO}_3$ , 10mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , pH 9.8) in a concentration of 1mg/ml, and the O.D.  
10 of the culture was determined by using an ELISA reader at 405nm. The amount of produced collagen was calculated from the O.D. value with reference to that of the internal control group.

As can be seen from Fig. 7, the amount of synthesized  
15 collagen decreases significantly in the culture of rat pulmonary fibroblast which was pretreated with acanthoic acid. In Fig. 7, si+PBS and si+acan. represent silica-stimulated sample and acanthoic acid-treated and silica-stimulated sample, respectively.

20        Further, for the purpose of determining the amount of synthesized collagen in rat pulmonary tissues, the amount of hydroxyproline was measured as follows.

0.1 to 0.2g of the rat pulmonary tissue was mixed with 1ml of PBS and then crushed in a Pyrex tube(Corning,  
25 Rochester, NY, U.S.A.). The resulting tissue extract was ruptured by using an ultrasonicator(Heat system, W-380), 1ml of hydrochonic acid was added thereto and the mixture was dried overnight at 120°C in a drying oven. The resultant was freezed in a freezer, lyophilized in a freeze-dryer(Labconco)  
30 and dissolved completely in 1ml of distilled water. 50 $\mu\text{l}$  of the resulting solution was diluted with 50 $\mu\text{l}$  of distilled water in a microcentrifuge tube. As an internal control group, trans- $\gamma$ -hydroxy-L-proline(Sigma) was diluted in a concentration ranging from 20 $\mu\text{g}$  to 150pg, and 100 $\mu\text{l}$  of each  
35 diluted solutions was added to the microcentrifuge tube.

0.9ml of a solution prepared by dissolving 1.41g of chloramine-T(sodium N-chloro-P-toluene sulfonamide) in a

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mixture of 10ml of n-propanol and 10ml of distilled water was added to the tube, which was stored at a room temperature for 20 minutes. Then, to the resulting mixture was added 1ml of aldehyde/perchloric acid solution prepared by dissolving 15g  
5 of p-dimethyl aminobenzaldehyde in 62ml of n-propanol and then adding 26 ml of 60% perchloric acid thereto to make the total volume of 100 ml, and the resultant was mixed well. The microcentrifuge tube was reacted at 65°C in a water bath for 15 minutes to develop colors. O.D. of the sample was measured  
10 at 650nm, and the amount of hydroxyproline in the sample was calculated by employing the standard curve of internal control group.

As can be seen from the result in Fig. 8, when the amount of collagen produced in normal rat pulmonary tissue(normal) is  
15 regarded as 100%, the amount of collagen synthesized in rat pulmonary tissue treated with silica only(si+PBS) or treated with silica and dimethylsulfoxide(si+DMSO) is remarkably high, while the amount of synthesized collagen decreases by about 50% in rat pulmonary tissue treated with silica, DMSO and  
20 acanthoic acid(si+acan.). The above result shows that acanthoic acid has anti-fibrogenic activity.

Example 7: Inhibition of IL-6 production by Acanthoic Acid

25 For the purpose of confirming the in vivo anti-fibrogenic activity of acanthoic acid, an experimental silicosis model was established and the effect of acanthoic acid on the silicosis model when administered thereto was determined.

Silica was refined by removing any contaminant, e.g.,  
30  $\text{Fe}_2\text{O}_3$ , therefrom in accordance with the method of Lugano as described in Am. J. Pathol., 109, 27-36(1982) as follows. Silica powder(Sigma, St. Louis, MO; the content of the particles having a diameter of 5 $\mu\text{m}$  is at least 80%) was suspended in 1N HCl and the suspension was heated and washed  
35 with distilled water. The resultant was sterilized by dry heating at 200°C for 2 hours and then used in the following experiment.

- 20 -

A 7 to 8 week-old male Sprague-Dawley rat and a 5-week old female ICR mouse were anesthetized by an intraperitoneal injection of 2 to 5mg of ketamine chloride. The rat was injected into their bronchia with 50mg of silica dissolved in 0.5ml of sterilized PBS and the mouse was injected with 2 mg of silica dissolved in 0.1ml of sterilized PBS, by using an 1ml syringe. From the next day after the injection of silica, 10 mg of acanthoic acid was administered orally to the rat and mouse twice a week for a period ranging from 12 to 18 weeks.

Thereafter, the lungs of the rat and mouse were separated and fixed in 10% neutral formalin, spread out in 4mm thickness and then embedded in paraffin in accordance with a conventional method. The embedded tissue was sectioned in 5mm thickness, stained with hematoxylin eosin, Masson's trichrome and reticulin, and then observed under a microscope(Fig. 9).

As can be seen from Fig. 9, in the lung of the rat administered with silica and DMSO(A), numerous coalescent granuloma showing excessive fibrosis and connective tissue formation, excessive monocyte infiltration, fibrosis and hyalinization are observed, while only small-sized granulomas which is not coalesce with each other and a slight fibrosis are observed in the lung of the rat administered with silica, DMSO and acanthoic acid(B). This result shows that acanthoic acid has an ability to inhibit the experimental silicosis.

25

#### Example 8: Inhibition of Hepatocirrhosis by Acanthoic Acid

Hepatocirrhosis(hepatic sclerosis) is characterized by the fibrosis of whole liver, complete disruption of liver parenchyma by fibrous septa, and formation of regenerative nodules. It is derived mostly from a chronic hepatitis or chronic alcoholism, however, the precise causes thereof are unknown. In a hepatocirrhosis patient, the amount of cytokines, e.g., TNF- $\alpha$ , which is involved in inflammation and fibrosis, is in an increased state; and, therefore, the inhibition of hepatocirrhosis by acanthoic acid may be determined by the inhibitory activity to TNF- $\alpha$ .

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To induce the experimental hepatocirrhosis in accordance with the method of Nakataukasa, H., et al. as described in J. Clin. Invest., 85, 1833-1843(1990), 1.0ml/100g of body weight of CCl<sub>4</sub> solution(50% CCl<sub>4</sub>+50% corn oil) was injected  
5 intraperitoneally to 4 week old male Sprague-Dawley rats twice a week, and 0.2ml each of methanol extract of A. koreanum Nakai and acanthoic acid was administered orally at the time of the injection of CCl<sub>4</sub> twice a week. After 13 weeks from the start of the test, each of the rats was anesthetized with  
10 ether and the blood samples were obtained from the heart to determine serum glutamic-oxaloacetic transaminase(sGOT) value and serum glutamic-pyruvic transaminase(sGPT) value(Fig. 10).

As can be seen from Fig. 10, when compared with the blood sample obtained from the rat treated with CCl<sub>4</sub>, DMSO and PBS  
15 which was used as a control group, sGOT values of the blood samples obtained from the rats treated with each of methanol extract of A. koreanum Nakai(CCl<sub>4</sub>+A.M.) and acanthoic acid(CCl<sub>4</sub>+acan.) decreased by 20% and 30%, respectively. Further, sGPT values in the blood samples obtained from the  
20 rats treated with acanthoic acid(CCl<sub>4</sub>+acan.) decreased more than 40%.

For the pathohistological examination of the livers separated from the above rats, the liver was fixed in 10% aqueous solution of neutral formalin, spread out in 4mm-  
25 thickness and then embedded in paraffin. The embedded tissue was sectioned in 5mm thickness, stained with hematoxylin eosin and Masson's trichrome, and then observed under a microscope (Fig. 11).

As can be seen from Fig. 11, in the liver of the rat administered with CCl<sub>4</sub> only(B, C and D), the nodule formation of hepatic lobules with the relatively thickened fibrous bands is remarkable compared with the normal liver(A). In the liver of the rat administered with CCl<sub>4</sub> and methanol extract of A. koreanum Nakai(E), even though signs of hepatocirrhosis are  
35 shown, their fibrous bands surrounding the nodule of hepatic lobule are thinner than those of the liver obtained from the rat treated with CCl<sub>4</sub> only, many nodules are incomplete, and

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the regenerative change of hepatic cells decreases compared with that of the liver obtained from the rat treated with  $\text{CCl}_4$  only. In the liver of the rat administered with  $\text{CCl}_4$  and acanthoic acid(F), the inhibitory effect on the  
5 hepatocirrhosis is lower than that of E.

The following Formulation Example is for illustration only and not intended to limit the scope of the invention in any way.

10

Formulation Example

Hard gelatin capsules were prepared using the following ingredients:

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	Quantity (mg/capsule)
Active ingredient	20
Starch, dried	160
<u>Magnesium stearate</u>	<u>20</u>
20 Total	200 mg

The above ingredients were mixed and filled into hard gelatin capsules in 200mg unit quantities.

25

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made and also fall within the scope of the invention as defined by the claims that follow.



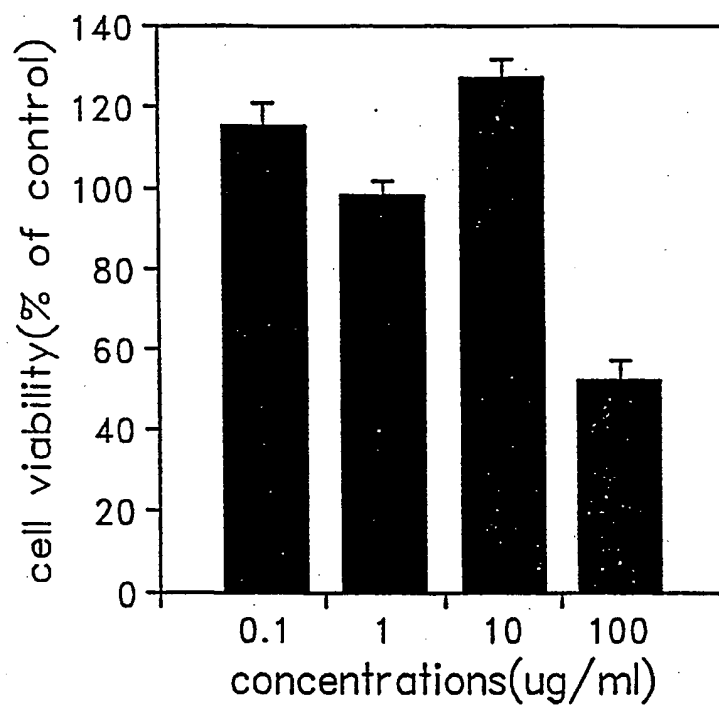
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What is claimed is:

1. A pharmaceutical composition comprising a therapeutically effective amount of acanthoic acid and a pharmaceutically acceptable carrier, for the treatment of an immune disease caused by an excessive production of interleukin-1 or tumor necrosis factor- $\alpha$ .
2. The pharmaceutical composition of claim 1 wherein said immune disease is caused by collagen synthesis, production of reactive oxygen species, proliferation of fibroblast or increase in GOT and/or GPT levels in serum.
3. The pharmaceutical composition of claim 1 wherein said disease is septicemia, inflammation, rheumatoid arthritis, hepatocirrhosis or silicosis.
4. The pharmaceutical composition of claim 1 wherein said acanthoic acid is extracted from Acanthopanax koreanum Nakai.
5. The pharmaceutical composition of claim 4 wherein said acanthoic acid is extracted with an organic solvent.
6. A process for the preparation of acanthoic acid which comprises the steps of: adding methanol to the root bark of Acanthopanax koreanum Nakai; heating the mixture at a temperature ranging from 20 to 60°C for a period ranging from 2 to 8 hours or at a room temperature for at least 10 hours; filtering the heated mixture to obtain a filtrate; concentrating the filtrate to obtain a methanol extract; partitioning said methanol extract with water and diethyl ether to obtain a diethyl ether fraction; concentrating said diethyl ether fraction to obtain a concentrate; purifying the concentrate by a silica gel column chromatography using a mixture of hexane and ethyl acetate as an eluent.

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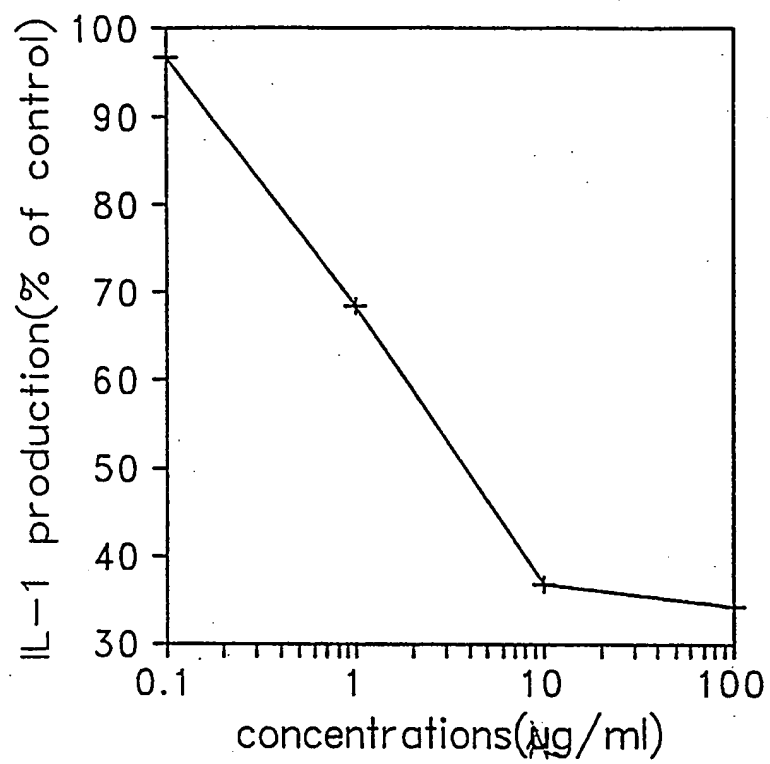
Fig. 1



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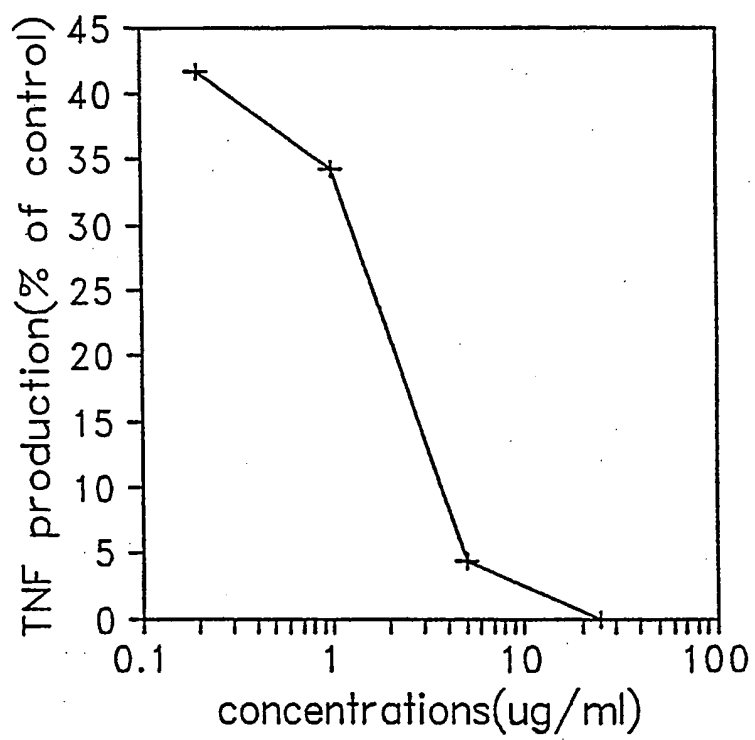
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Fig. 2



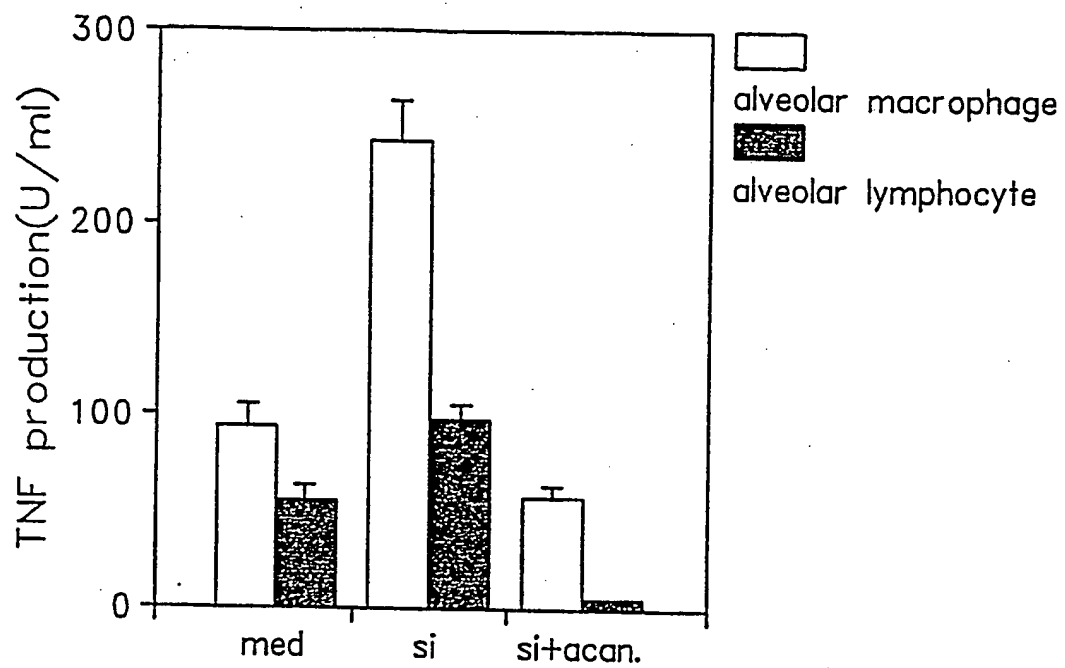
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Fig. 3



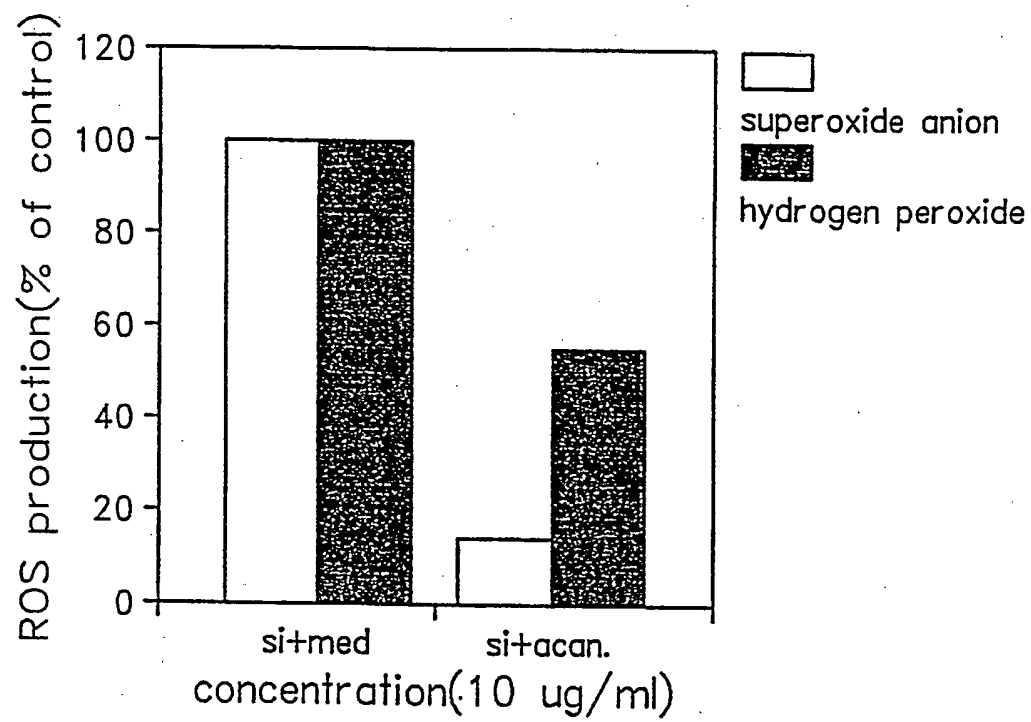
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Fig. 4



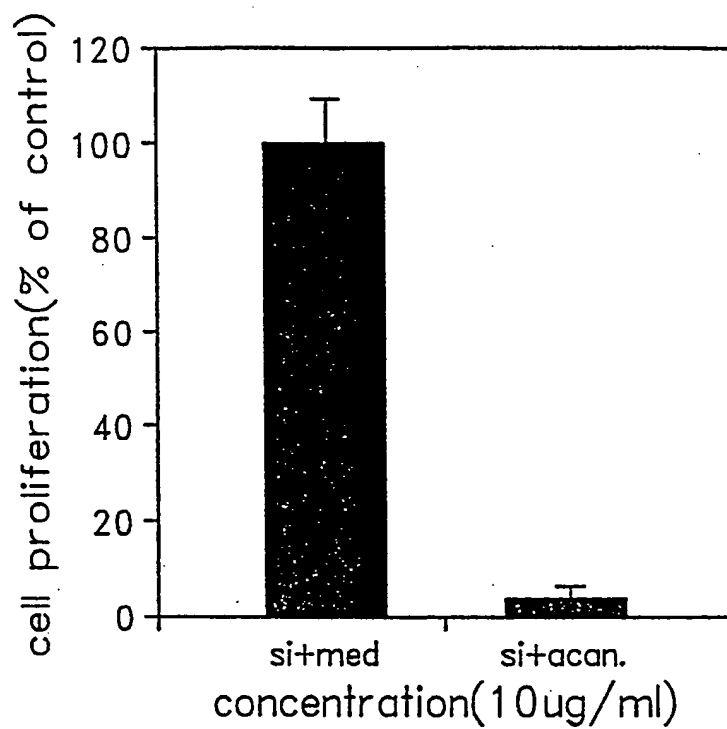
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Fig. 5



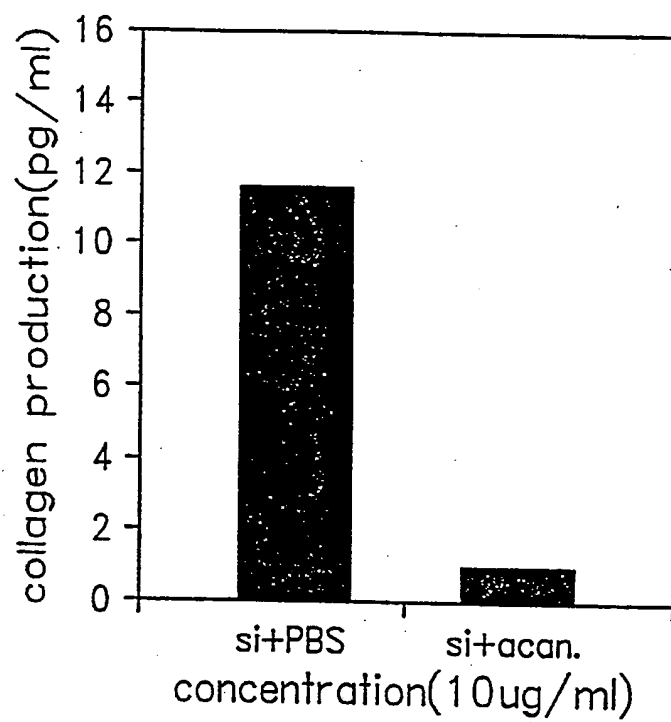
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Fig. 6



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Fig. 7

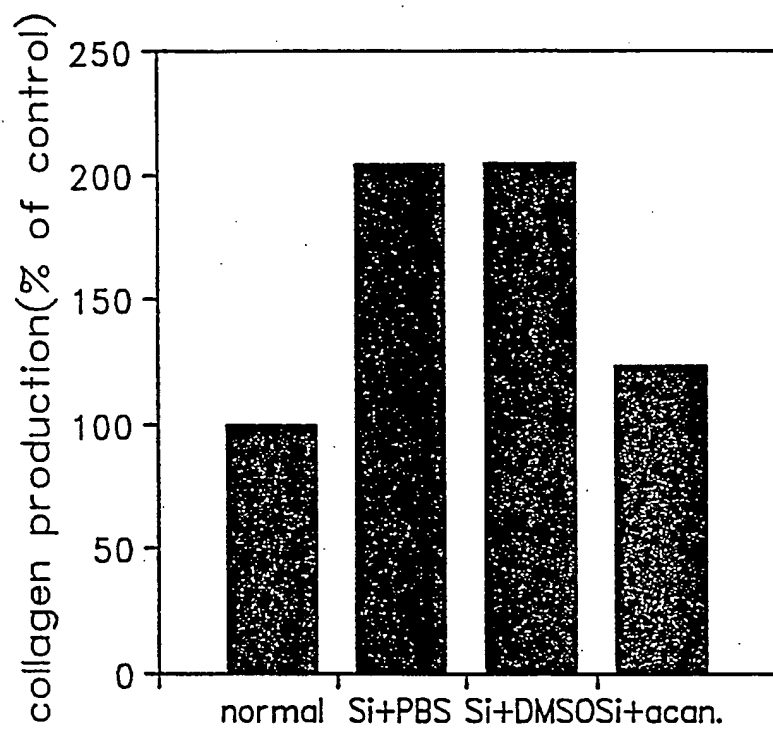


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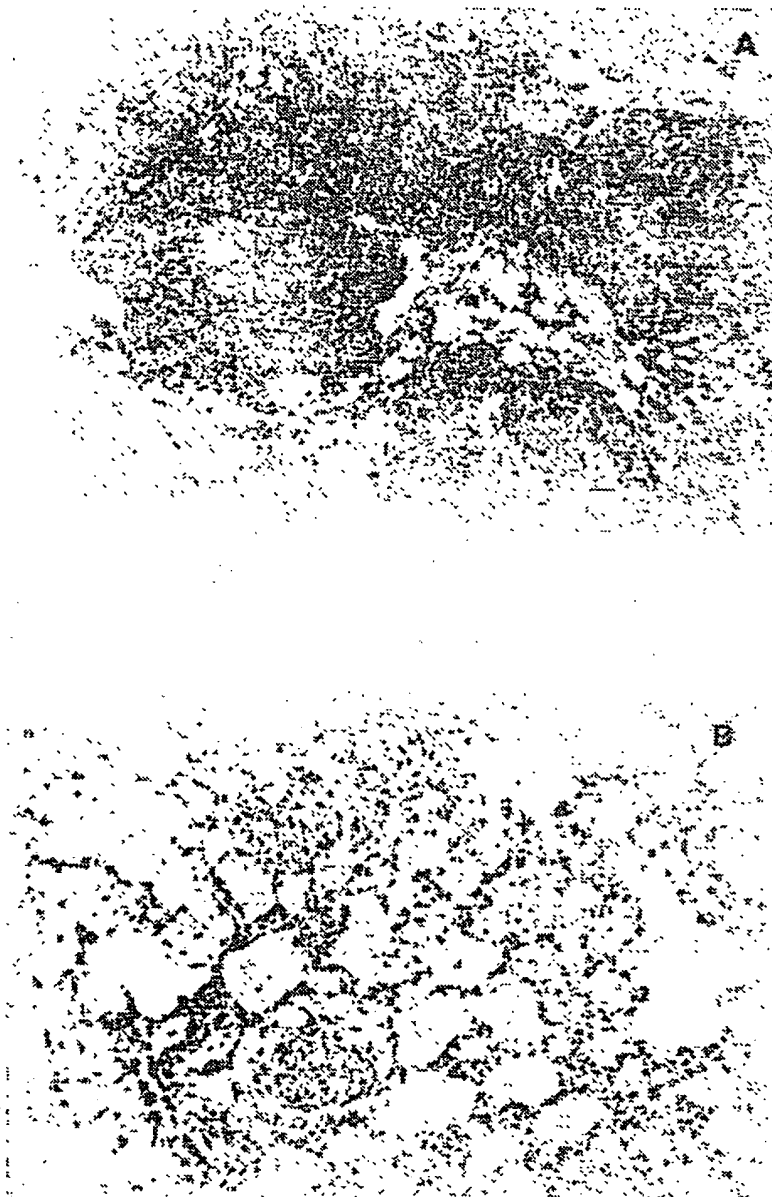
Fig. 8



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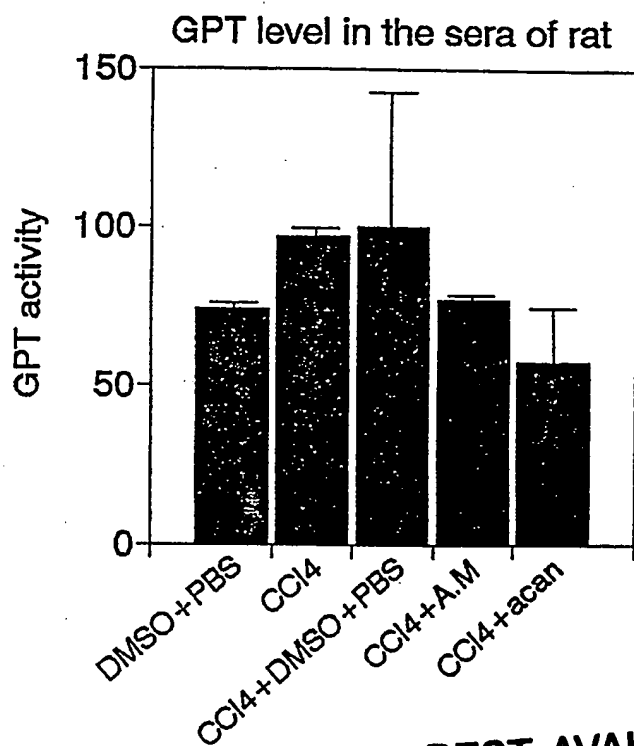
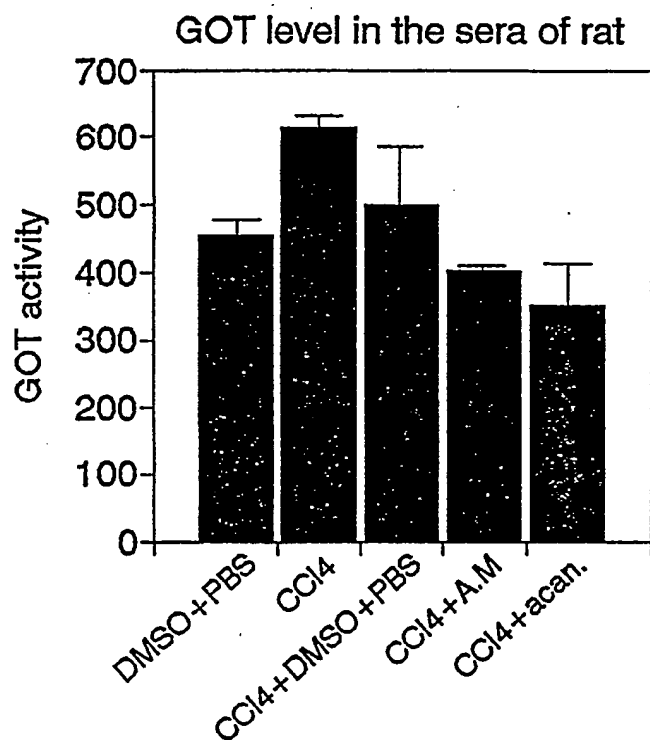
Fig. 9



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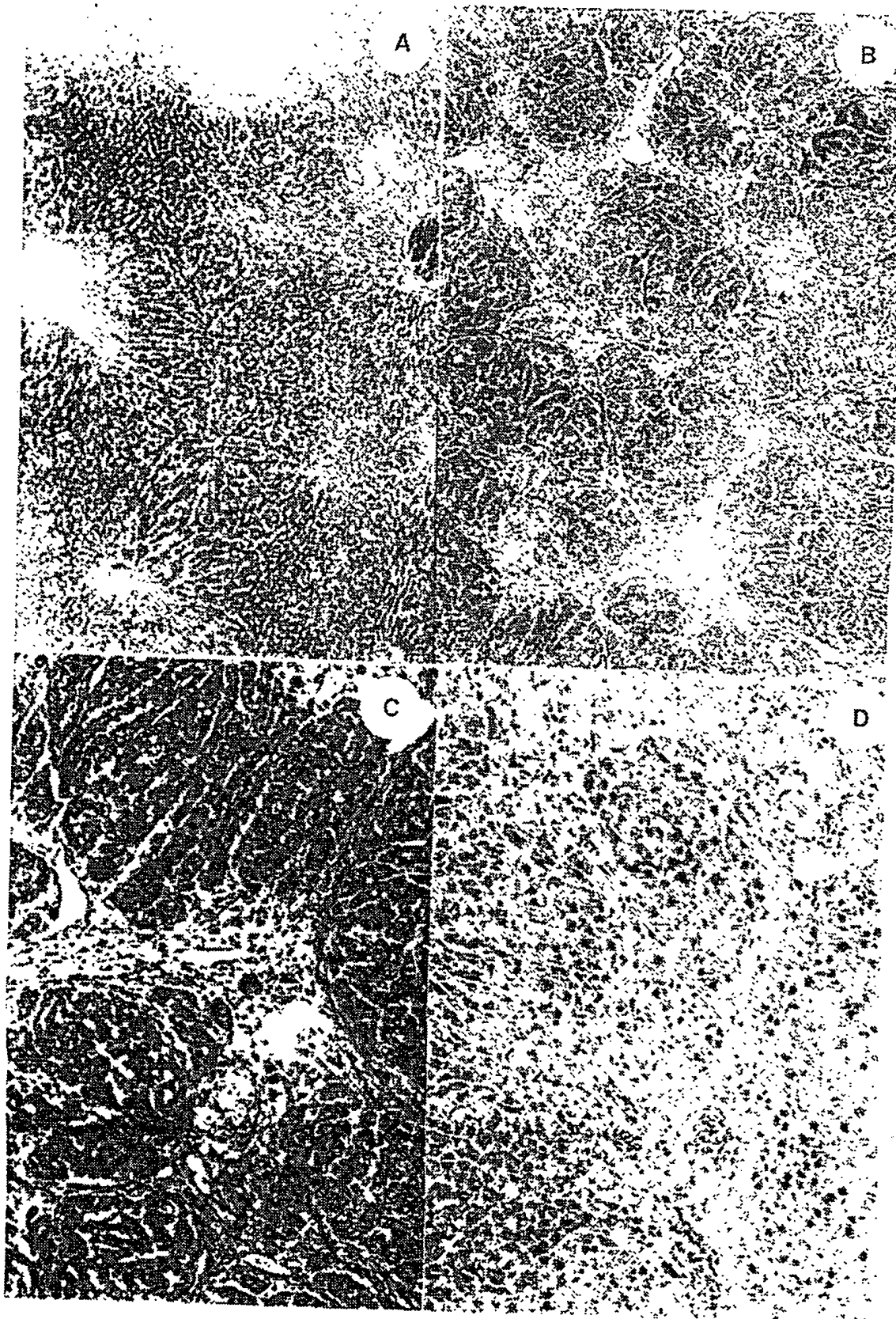
Fig. 10



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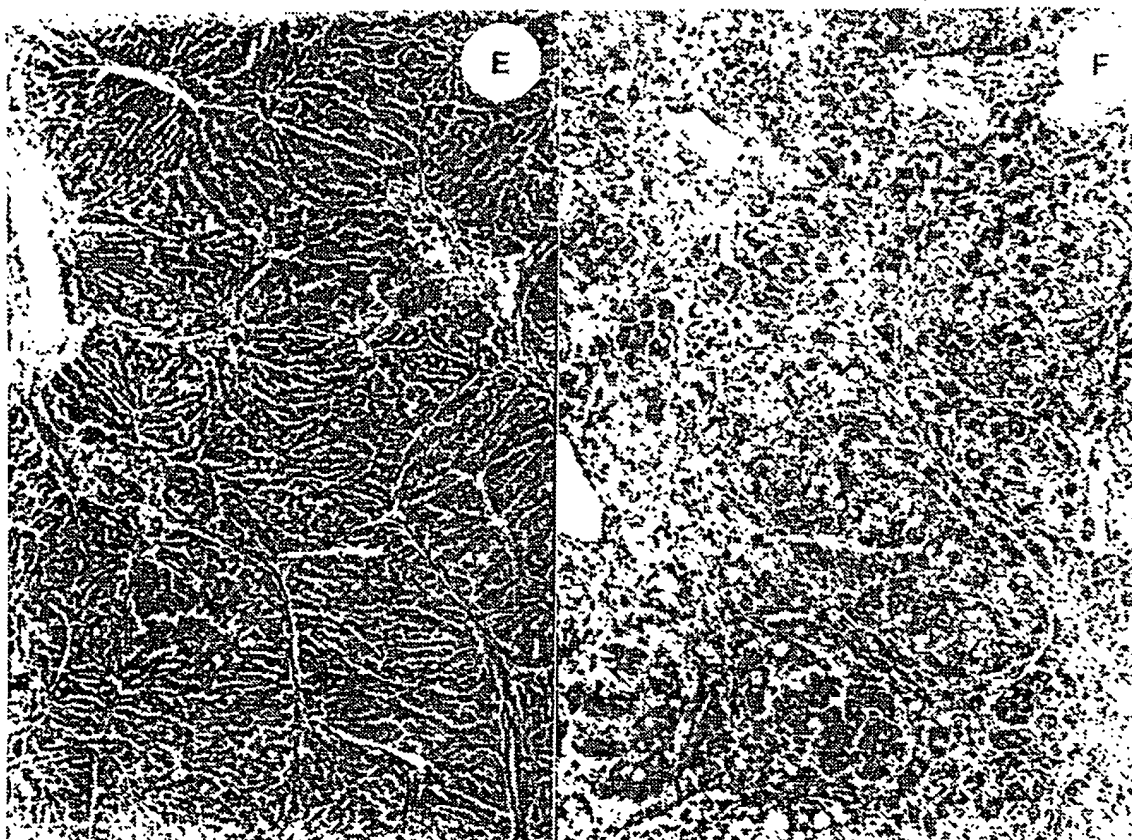
Fig. 11



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Fig. 11 (Continued)



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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 95/00074

## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>6</sup>: A 61 K 31/19, 35/78

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>6</sup>: A 61 K 31/19, 35/78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIM Y.H., CHUNG B.S., Pimaradiene Diterpenes from <i>Acanthopanax Koreanum</i> , Journal of Natural Products, 1988, vol. 51, no. 6, pages 1080-1083; page 1080, paragraphs 1-3, compound no. 2, page 1082, paragraph "Extraction and Isolation" (cited in the application).	1-6
A	BLADT S. et al., Taiga-Wurzel, Deutsche Apotheker Zeitung, 1990, vol. 130, no. 27, page 1499-1508; pages 1499-1500, abstract, paragraph "Pharmakologie und Klinik", especially page 1500, right column, lines 9-20, page 1505, right column, line 9 - page 1056, left column, line 8.	1-5
A	BOHN B. et al., Flow-cytometric Studies with <i>Eleutherococcus Senticosus</i> Extract as an Immunomodulatory Agent, Arzneimittel Forschung/Drug Research, 1987, vol. 37(II), no. 10, pages 1193-1196, page 1193, summary, introduction, pages 1195-1196, results, discussion.	1-5

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>
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Date of the actual completion of the international search  
29 August 1995 (29.08.95)

Date of mailing of the international search report  
12 September 1995 (12.09.95)

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 95/00074

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chem. abstr. vol. 108, no. 9, 29 February 1988 (Columbus, Ohio, USA), page 40, column 2, abstract-no. 68519e, SADYKOV S.B. et al. "Effect of Eleutherococcus senticosus extract on the T-system immune response during stress", Zdravookhr. Kaz. 1987, (11), 52-5 (Russ).	1-5
A	Chem. abstr. vol. 111, no. 23, 4 December 1989 (Columbus, Ohio, USA), page 58, column 1, abstract-no. 209123x, TAKAHASHI T. et al. "Inhibitory effects of constituents of Acanthopanax senticosus on lipid peroxidation in rat liver microsomes", Hokkaidoritsu Eisei Kenkyushoho 1989, (39), 94-7 (Japan).	1-5
A	WO 91/19 507 A1 (CEDARS-SINAI MEDICAL CENTER) 26 December 1991 (26.12.91), claims 7, 24, 30, 37; page 5, table 1.	1-5
A	ONO K. et al., Differential Inhibitory Effects of Various Herb Extracts on the Activities of Reverse Transcriptase and Various Deoxyribonucleic Acid (DNA) Polymerases, Chem. Pharm. Bull., 1989, vol. 37, no. 7, pages 1810-1812, page 1811, table 1, plant no. 17.	1-5
A	US 5 248 696 A (BANG S.D. et al.) 28 September 1993 (28.09.93), claims 1-3.  -----	1-5

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.

PCT/KR 95/00074

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9119507	26-12-91	US A 5178865 AP A0 9100304 AP A 233 AU A1 82038791 AU B2 644669 CA AA 2064743 EP A1 491913 EP A4 491913 JP T2 5500824	12-01-93 31-07-91 07-03-81 07-01-92 16-12-93 20-12-91 01-07-92 17-02-93 18-02-93
US A 5248696	28-09-93	AU A1 18886/92 WO A1 9218119	17-11-92 29-10-92